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STUDIES OF THE BIOLOGICAL AND MOLECULAR BASIS OF THE INHIBITION OF ACTIVITY OF PHAGOCYTIC CELLS BY ANTHRAX TOXIN

Annual Report

George G. Wright, Paul W. Read,
and Gerald L. Mandell

April, 1987

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Grant No. DAMD17-83-G-9565

Division of Infectious Diseases
Department of Internal Medicine
The University of Virginia
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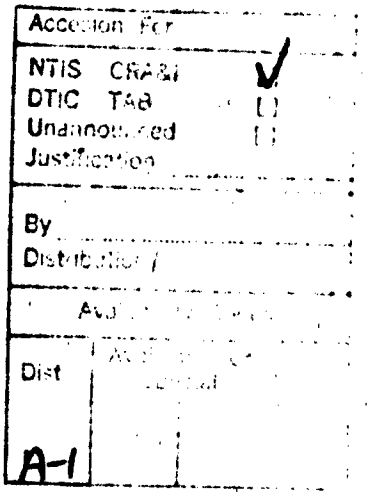

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Resting human neutrophils produce only small amounts of superoxide anion when stimulated with chemotactic peptides; preincubation with low concentrations of lipopolysacchafide (LPS) markedly increases this response, an effect referred to as priming. Priming is inhibited by anthrax toxin, an action presumably related to the critical role of the toxin in virulence of Bacillus anthracis. Priming has now been found to be mediated by platelets, and to involve action of a labile factor which is released from platelets by LPS. We present observations on conditions for release and activity of priming factor, on its chemical nature, and on the inhibition of its action by anthrax toxin. Keywords:)

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Summary

Resting human PMN, partially purified without exposure to LPS, produce small amounts of O_2^- when stimulated with the chemotactic peptide FMLP; preincubation of the PMN with low concentrations of LPS markedly increases this response, an effect referred to as priming. Further purification of the PMN on Percoll gradients removed most remaining mononuclear cells and platelets, yielding PMN preparations approximately 98% pure. We found that these PMN suspensions were not susceptible to priming by LPS; susceptibility was restored to a major degree by reintroduction of platelets, approximately 5 per PMN.

Incubation of platelets, isolated without LPS exposure, with LPS at concentrations of the order of 10 ng per ml released a soluble factor that produced priming responses in PMN of at least five-fold. The priming factor had properties of a labile protein, and did not resemble previously described mediators derived from platelets. It was non-dialyzable, did not pass an ultrafilter with 30,000 Dalton cut-off, and was precipitated by 40% saturation with ammonium sulfate. Activity of the crude filtrate was destroyed immediately at pH 5 or below; moderate activity was retained after brief exposure to pH 10. Efforts to extract priming activity in lipid solvents gave negative results. Anthrax toxin, previously shown to inhibit priming of PMN by LPS, also inhibited priming of PMN by platelet-derived priming factor, but had no evident effect on release of priming factor from platelets.

Evidently platelet-derived priming factor mediates a portion of the overall priming effect of LPS described previously, thereby modulating the level of O_2^- generation by PMN.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

(For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR4)

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Introduction

Evidence has accumulated that phagocytic cells in the resting state produce relatively weak oxidative responses and granule exocytosis when exposed to chemotactic peptide and certain other conventional stimuli. In the case of polymorphonuclear neutrophils (PMN), and macrophages, pretreatment with low concentrations of bacterial lipopolysaccharide (LPS) produces a major increase in responsiveness, an effect referred to as priming (1-5). Muramyl dipeptide (MDP), a synthetic mitogen related in structure and activity to peptidoglycans of bacterial cell walls, produces similar priming effects, indicating that priming may occur in response to products of Gram positive as well as Gram negative bacteria (5).

The critical contributions of the respiratory burst and the release of granule contents to the antimicrobial activity of phagocytes makes it probable that priming which augments the reactions is essential for normal functioning of these cells in host defense; this notion is supported by the observation that anthrax toxin, an essential virulence factor of Bacillus anthracis, inhibits priming of PMN (5). Priming also increases the potential of PMN to damage adjacent normal tissue, and may contribute to the etiology of lesions of endothelium and other tissues (6-8). Priming is a relatively slow process, requiring about 1 hour to approach completion in vitro at 37° C (1). Guthrie et al. reported that priming did not increase binding of FMLP to receptors, nor involve synthesis of protein; it was associated with an increased V_{max} of the

NADPH oxidase, suggesting that activation of the oxidase was more efficient in primed PMN (1). Further understanding could provide concepts useful in therapeutic modulation of priming, either stimulation to bolster host defense, or inhibition when priming is inappropriate or excessive.

Our experiments on priming and its inhibition by pretreatment of PMN with anthrax toxin (5) were carried out with cells isolated by a simplified method involving dextran sedimentation and hypotonic lysis of erythrocytes, procedures selected to avoid uncontrolled introduction of the minute concentration of LPS sufficient for spontaneous priming (1,2). The suspensions contained about 20% mononuclear cells; platelets were not enumerated but were present in considerable numbers. In an effort to avoid ambiguities associated with use of this non-homogeneous cell preparation, exploratory experiments were carried out with PMN isolated on Percoll density gradients (9). Despite the presence of small amounts of LPS in Percoll, as indicated by the Limulus Amoebacyte Lysate Test, the isolated PMN did not prime spontaneously; indeed they were relatively resistent to priming by added LPS.

The present paper reports restoration of susceptibility to LPS priming in these purer PMN suspensions by reintroduction of platelets, the elaboration of a soluble priming factor for PMN by action of LPS on platelets, and certain chemical and biological properties of the priming factor, suggesting that the factor is a protein. Evidence is presented that the inhibitory effect of anthrax toxin on priming is exerted not on elaboration of priming factor by platelets, but on its action on PMN.

Materials and Methods

Preparations of PMN. Two types of preparations were used: 80% PMN were prepared from citrated normal human blood by dextran sedimentation and hypotonic lysis of erythrocytes as described previously (5), except that a modified Hank's Balanced Salt Solution (HBSS) was used, and changes were instituted to reduce the carry-over of platelets. Supernatants from dextran sedimentation of erythrocytes containing PMN and platelets were centrifuged at 100g for 15 minutes to sediment PMN; the platelet-rich plasma supernatant was used for isolation of platelets. In addition, the speed of centrifugation of PMN suspension during supernatant washing was reduced to 150g. For 10 consecutive lots of PMN the mean number of platelets/PMN was 2.95 ± 0.36. HBSS modified by omission of magnesium sulfate and phenol red, and by addition of 0.01 M HEPES buffer, was used throughout except for isolation of platelets (see below); it is referred to as HMN. Preparations of HEPES frequently were too pyrogenic for use, however a product HEPES Buffer IM, Cell Culture Tested, supplied by Sigma Chemical Co., proved sufficiently low in LPS. 98% pure PMN were isolated on Percoll gradients (9) and contained no detectable platelets. Absolute numbers of PMN in 98% pure PMN preparations were approximately half those obtained with 80% PMNs.and these cells were used, as indicated, only in experiments in which absence of platelets was critical.

Preparation of Platelets. The method of Lagarde et al (10) was used, except that the platelet pellet was resuspended in HMM without calcium chloride. A 1:100 dilution was prepared in buffered ammonium oxalate (Unopette Microcollection System, Becton Dickinson, Rutherford, N.J.) and

platelets were counted in a platelet counting chamber under phase contrast at a magnification of 400X.

Toxins. PA, EF, and LF were supplied by Dr. S.W. Leppla of the U.S. Army Medical Research Institute of Infectious Diseases, Frederick MD, and resembled previous lots in biological activity (11). LPS from Escherichia coli K235 was obtained from List Biological Laboratories, Campbell, CA. MDP was obtained from Sigma Chemical Co.

Production of Priming Factor

Measurements of Priming. The extent of priming of PMN was determined from the increase in release of superoxide (O_2^-) relative to unprimed controls following stimulation with 10^{-7}M FMLP. O_2^- release was determined by spectrophotometric measurement of reduction of cytochrome C as described in detail elsewhere (5).

Physical Manipulations. Ultrafiltration was carried out with Centrifree Minifiltration System, produced by Amicon Corp., Danvers, MA. The molecular weight cutoff for the membrane in this device is 30,000 Daltons. Dialysis was carried out in short sections of 5/8" D. Dialysis Tubing, obtained from VWR Scientific, Bridgeport, NJ. Ultracentrifugation:a 4ml sample of priming factor was centrifuged at 100,000 g for 1 hour at 4°C. The upper 1.0 ml was removed for titration of priming activity.

PH adjustment and neutralization. These were performed by dropwise additions from a Pasteur pipette of M/100 HCl or M/100 NaOH to 1 ml portions of priming factors.

Results

Priming of PMN Isolated on a Density Gradient; Reintroduction of Platelets. The effect of pretreatment of 98% pure PMN for 1 hour at 37° C with a range of concentrations of LPS on release of superoxide anion (O_2^-) after stimulation with 10^{-7}M FMLP was investigated. No appreciable priming effect of LPS treatment was observed at 1 and 10 ng/ml, and only a slight effect was obtained at 100 ng/ml (Figure 1). This was in sharp contrast with prior results using 80% PMN, in which 1 ng per ml of LPS produced strong priming. A consistent difference between the 98% pure PMN and the 80% PMN preparations used in the previous study (5) was the virtual absence of platelets in the former. The effect of introduction of platelerts on the priming reaction was studied. Addition of 1, 5, or 25 platelets per PMN prior to addition of LPS produced progressive increases in priming (Fig. 1). Evidently platelets play a critical role in priming of PMN by LPS.

Release of a Priming Factor from Platelets by LPS. The foregoing results were compatible with the hypothesis that platelets contained a priming factor for PMN that was released by LPS. Exploratory experiments confirmed this notion and revealed suitable conditions for preparation of a soluble priming factor. Platelet suspension was mixed with an equal volume of diluted LPS to give final concentrations of platelets ranging, in various experiments, from 1- to 5 x 10^8 per ml, and LPS ranging from 1 ng to 100 ng per ml. The mixtures were incubated for 30 min at 37° C in a shaker

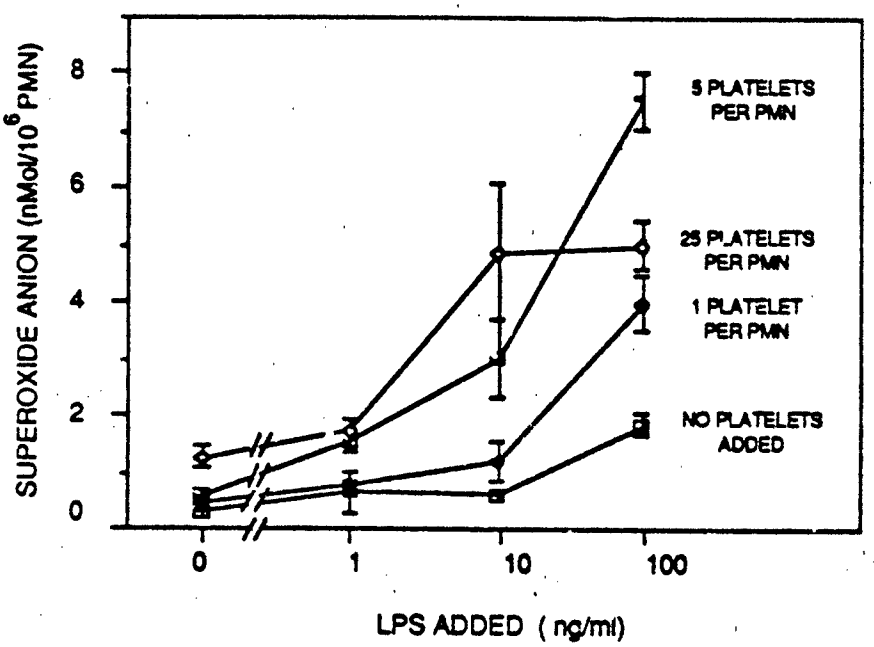

Figure 1. Release of O_2^- after stimulation of PMN with 10^{-7} FMLP as a function of concentration of LPS and number of platelets per PMN during priming. 98% pure PMN suspensions isolated on Percoll gradients were used. The mixtures of PMN, platelets, and LPS was incubated for 1 hour at 37°C, stimulated with FMLP, and the O_2^- released in 10 minutes measured. n = 4.

water bath. Platelets were sedimented by centrifugation for 15 minutes at 2000g and the supernatants tested for priming activity for PMN. Results with representative preparations of priming factors are presented in Fig. 2.

Although release of priming activity was detectable after exposure of platelets to 1 or 3 ng/ml of LPS, 10 ng/ml appeared most suitable since it produced essentially maximum priming activity without leaving a residual concentration of LPS that was itself capable of priming PMN. Higher concentration of platelets or LPS seemed to give no advantage in priming activity per platelet (not shown). Observations on the influence of the time of incubation of priming factor and PMN on subsequent release of O_2^- after stimulation with FMLP are presented in Fig 3. Priming was essentially complete in 20 minutes under these conditions. This interval was used in subsequent work because it minimized any priming of PMN by LPS itself; this reaction requires 60 minutes or more to reach substantial completion (1).

Release of Priming Factor from Platelets by MDP. MDP at concentrations of 10-30 times those used for LPS had yielded almost equal priming of the 80% PMN suspensions used in the previous studies (5). However, treatment of platelets with a range of concentrations of MDP up to 20μg/ml gave priming activity less than 1/4 that obtained with 10 ng/ml LPS.

Properties of Crude Priming Factor. Results of experiments that provide insight into the nature of priming factor and indications of its stability under various conditions are summarized in Table I.

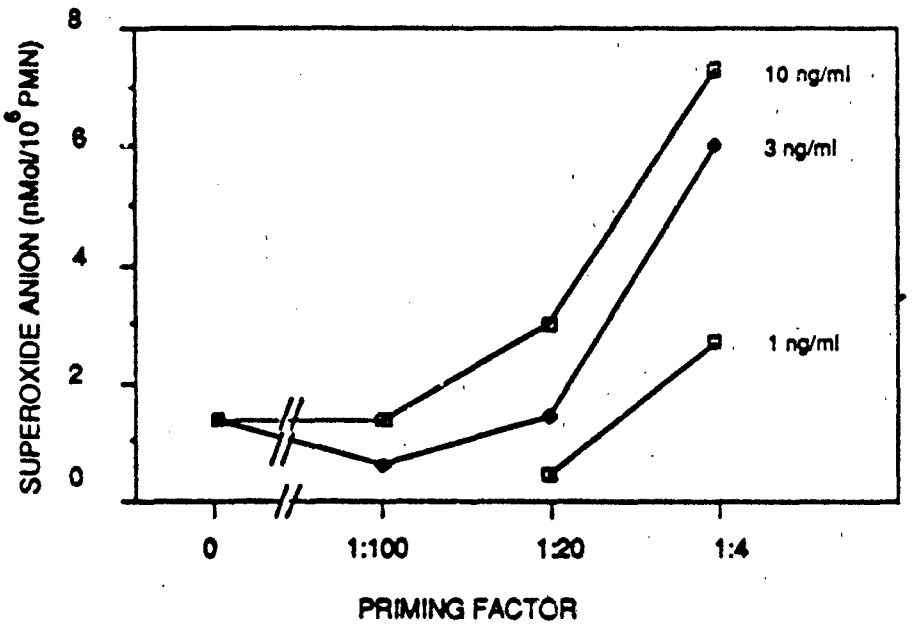

Figure 2. Release of O_2^- after 20 minute incubation of PMN with preparations of priming factor, each at several final dilutions (abcissa), followed by stimulation with FMLP and measurement of O_2^- release as before. The initial concentration of platelets was 10^8 per ml in each case; the concentrations of LPS used for release of priming factor are shown on the right of the figure. n = 2.

Table 1

Stability of Priming Factor to Various Treatments as Measured by Change in Release of O_2^- by Treated PMN after FMLP Stimulation

Treatment	Activity of Treated Priming Factor Relative to Untreated Priming Factor %
-70°C, rapid thaw	108
30 K-dalton ultrafiltration (filtrate tested)	0
dialysis (20 hours at 4°C)	136
100,000g for 1 hr (supernatant)	102
pH 10, neutralize	66
pH 5, neutralize	2
pH 3, neutralize	1
60°C for 5 minutes	31
70°C for 5 minutes	2

Although the activity was relatively unstable at 4°C, losing perhaps half its activity in 24 hours, the activity resisted freezing at -70° and rapid thawing at room temperature. Preparations have been held at -70° C for several months without evident loss. The activity did not pass an ultrafilter with 30,000 Dalton cutoff and was retained in cellophane dialysis tubing; indeed brief dialysis in the cold showed a small but consistent tendency to increase the activity, suggesting the presence of a dialyzable inhibitor of priming in the crude factor. The activity was unchanged after ultracentrifugation, suggesting that it is not associated with fragments of platelet membranes. The activity proved to be moderately stable to alkali, but unstable to acid, even momentary adjustment to pH5 destroying it. Efforts to extract priming activity into ethyl acetate (12), diethyl ether (13), or chloroform gave negative results.

The priming activity proved to be precipitable with ammonium sulfate at a concentration of 0.4 saturation. In a typical experiment, the dry salt was dissolved in crude platelet factor and the mixture held 15 minutes at 0°C. A slight turbidity developed which was sedimented at 3000g for 10 minutes in a fixed angle centrifuge, yielding a barely visible pellet. The supernatant was poured off and the tube wiped. The pellet dissolved readily in chilled HMM buffer containing 0.2% human serum albumin. The product was dialyzed for 2 hours at 4°C in cellophane tubing against several changes of HMM to remove residual ammonium sulfate. Results of assays for priming activity (Fig. 4) indicate that 20-25% of the activity was recovered during the precipitation.

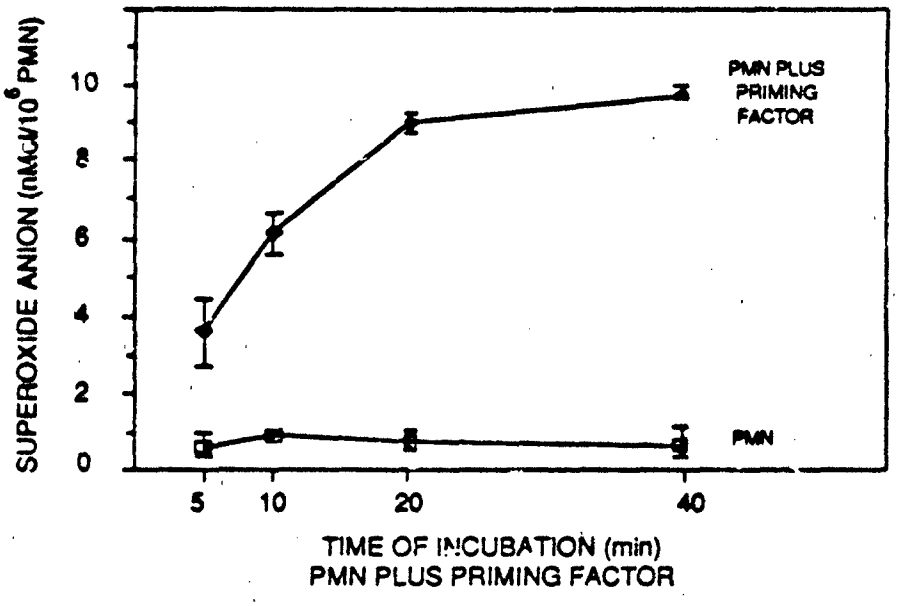

Figure 3. Effect of incubation time at 37°C of priming factor plus PMN on subsequent release of O_2^- after stimulation with FMLP. n = 2

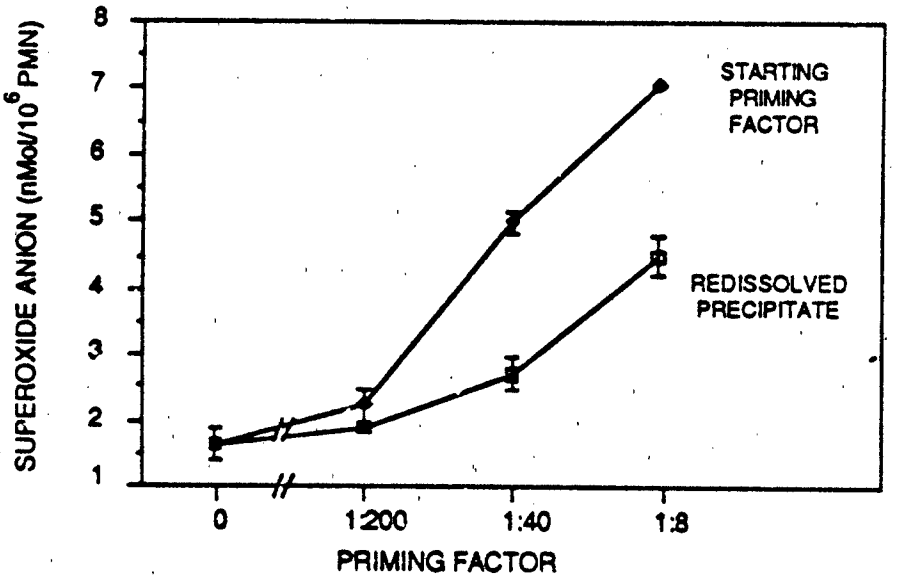

Figure 4. Comparison of activity of priming factor before and after precipitation with ammonium sulfate, as measured by O_2^- release by treated PMN, n = 2.

Site of Action of Anthrax Toxin in Inhibition of Priming. It was of interest to determine which component of the overall priming effect was inhibited by anthrax toxin (5): the release of priming factor from platelets, or an action of priming factors on PMN. Multiple portions of platelet suspension at a final concentration of 10^8/ml were exposed to PA plus EF (each 0.25µg/ml final concentration) or to control HMM, for 30 minutes at 37° C. HMM or LPS diluted to yield final concentrations of 0.4, 2, and 10 ng/ml was then added, and incubation was continued for 15 minutes. Platelets were sedimented at 2000g, and the supernatants were tested for priming activity at final dilutions of 1:40 and 1:8. The conditions were selected in an effort to favor expression of inhibitory effects of toxin should they be present. The results (Fig. 5) gave no indication of any effect of PA plus EF on release of priming factor from platelets.

Methods developed previously to establish the inhibitory effect of anthrax toxin on priming by LPS (5) were adapted to determine the effect of the toxin on priming by a series of concentrations of priming factor. The results (Figure 6) indicate that pretreatment of PMN with either PA plus LF or PA plus EF inhibited priming by priming factor. PA plus EF was more effective than PA plus LF, as had been the case with priming by LPS (5).

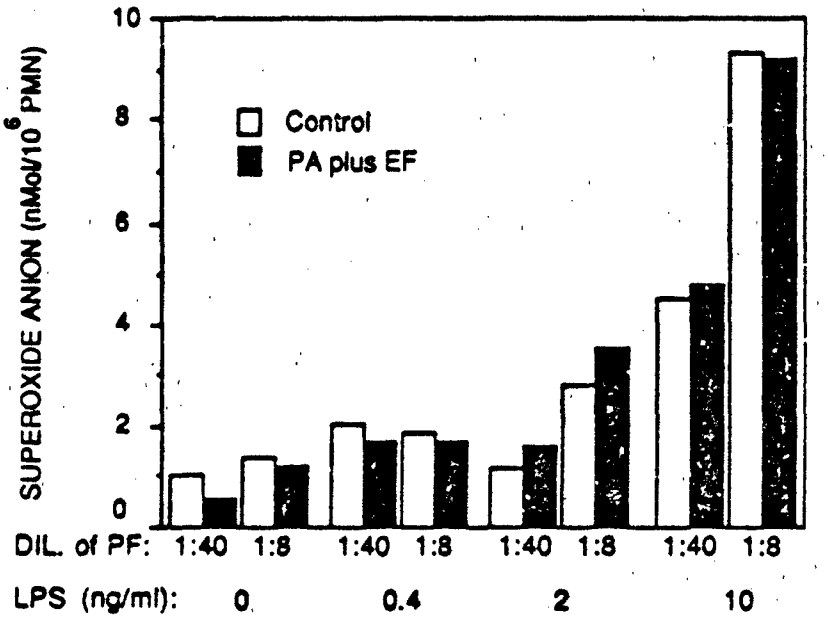

Figure 5. Effect of preincubation of platelets with PA plus EF on release of priming factor by a range of concentrations of LPS, as measured by incubation of the resulting priming factor preparation with PMN, and subsequent measurement of O_2^- release after stimulation with FMLP. n = 2.

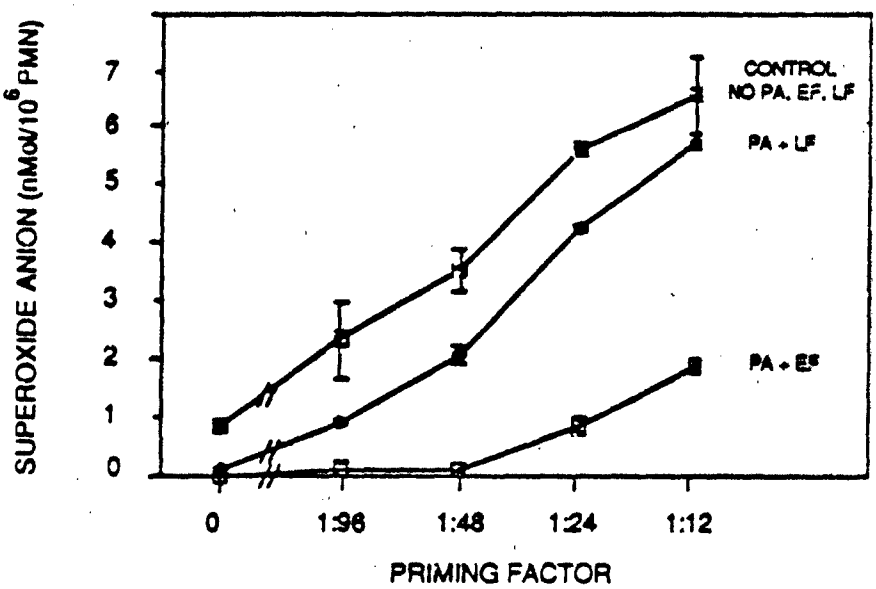

Figure 6. Effect of pretreatment of PMN with control buffer, with PA plus LF, or with PA plus EF, on release of O_2^- after subsequent priming with various dilutions of priming factor and stimulation with FMLP. n = 2.

Discussion

Reaction of washed human platelets with LPS is known to release serotonin and adenine nucleotides and to moderately increase their procoagulant activity (15,16). These products bear no evident relationship to the priming factor; their chemical properties are dissimilar and the concentration of LPS required to release them appeared to be greater by four orders than that required for release of priming factor. Relationship to platelet-derived growth factor also appears unlikely because of the stability of the latter to heat and acid.

Evidence has accumulated that platelets or products of platelets interact with PMN to produce changes in responsiveness suggestive of, or in some cases essentially identical with, priming by LPS. Goetzl et al (17) reported that treatment of PMN with 12-hydroperoxy-5-8-10-14-eicosatetraenoic acid (12-HPETE), the product of platelet arachnidonic acid lipoxygenase, raised the concentrations of guanosine-3'-5'-cyclic monophosphate and augmented release of lysosomal enzymes in response to C-5 fragments; 12-HETE, the breakdown product of 12-HPETE was substantially less active Maclouf et al. (18) reported that platelets or 12-HPETE, when added to PMN, modify their response to exogenous arachidonic acid by stimulating the 5 lipoxyglucose pathway and increasing the production of 5, 12-dihydroxyl-6-8-10-14 eicosatetraenoic acid, and leukotriene B_4 (LTB_4). The latter substances potentiate the response of PMN to FMLP, increasing the release of O_2^- (19-21). Vanderhoek et al. (14) found that pretreatment of PMN with 12-HETE inhibited the 5-lipoxygluase pathway and activated the 15-lipoxygenase , leading to formation of lipoxins and other active lipids

derived from 15-HPETE. 12-HPETE produced marked inhibition of the cyclooxygenase pathway in platelets and in macrophages (22-24). Clearly products of platelet 12-lipoxygluase have regulatory activity in PMN and various other cells, controlling the expression of powerful modulators of cellular function.

The hypothesis that priming factor was a product of platelet 12-lipoxygenase, reasonable on the basis of the regulatory activities just outlined, became untenable as the chemical properties of the substances became known; priming factor now appears to have the properties of a labile protein rather than those of a lipid. Efforts to further characterize it and the dialyzable component in crude priming factor that inhibits priming are in progress. A possibility that appears consistent with present information is the concept that priming factor is the enzyme arachidonate-12-lipoxygluase. Should this hypothesis prove correct, it would have important implications for efforts to control the level of priming; different strategies would be appropriate for control of a protein intercellular messenger than for a lipid. Whatever its nature, it is evident that priming factor is released from platelets by concentrations of LPS at least as low as 1 ng/ml.

Although the priming factor derived from platelets seems to make a major contribution to the priming reaction seen in previous work (5), there are several indications that it is not responsible for the total priming effect. First, platelets did not completely restore the susceptibility of pure PMN to LPS priming seen previously with the 80% PMN suspension, especially at the lowest concentration of LPS (Figure 1). Second, although priming by MDP in previous work required approximately 10-fold the concentration required for LPS, the levels of priming reached

by MDP and LPS were approximately the same. In release of priming factor from platelets, however, the activity of MDP did not approach that of LPS, even at concentrations 1000-fold greater. Third, priming by platelet-derived priming factor was not inhibited as effectively by anthrax toxin as in the original system; this was especially true for PA plus LF. These considerations support the concept that cells in addition to platelets are involved in mediation of the total priming effect in our original model (5).

Cytokines released by mononuclear blood cells in response to LPS and other stimuli increase the reactivity of PMN or other granulocytes as measured by cytotoxicity, antibody dependent killing, leukotriene biosynthesis, degranulation, and respiratory burst (25-30). Interleukin-1 has been recently reported to prime neutrophils (31). It seems probable that one or more cytokines from mononuclear cells will be found to act in concert with the platelet-derived priming factor to produce the total priming effect.

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